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Research Article

Isolating *Lysobacter enzymogenes* strains with enhanced protease activity via chemical mutagenesis

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Abstract

Proteases are the most important industrial enzymes which have attracted enormous attention due to their vast variety and well-defined specificity. Microbial proteases are superior to other sources like plant and animal proteases because of their desired characteristics for biotechnological application. In this regard, *Lysobacter enzymogenes* is a rich source for the production of antibiotics and proteases. However, strain improvement in order to obtain overproduced microorganisms is always demanded at an industrial scale. Therefore, in the present study in order to enhance *L. enzymogenes* protease production, random mutagenesis was applied using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as a chemical mutagen. Random mutagenesis was conducted on *L. enzymogenes* suspension cultivated on nutrient broth using different concentrations of NTG (100, 150, and 200 µg/ml) for 20 and 40 minutes. The treated bacteria were cultivated on nutrient agar containing casein as a selective media. Primary and secondary screenings were performed by measuring the diameter of the casein hydrolysis zones in the isolated bacteria and the related supernatants, respectively. Finally, the unit of protease activity was quantified by Anson's method of examining bacterial supernatants. Among the total of 30 isolated mutants, two mutants showed the highest level of extracellular proteolytic activity which showed 2.65 and 1.86 fold increments in contrast to the wild type, respectively. In general, the effect of mutagenesis by NTG can be emphasized to increase protease activity.

Introduction

Proteases, the largest group of hydrolytic enzymes account for approximately 60% of total worldwide marketed enzymes [1]. Since these enzymes play an important role in physiological processes, they can be extracted from immense sources such as animals, plants, and microorganisms [2]. However, microbial proteases are preferred for industrial applications due to the source availability and affordability, rapid growth rate, and ease of genetic manipulations [3,4]. *Lysobacter enzymogenes* which is known as a bio-control agent commonly produces many vital types of hydrolytic enzymes to control its environmental pathogens [5-8]. However, these bacteria release the enzyme only to the extent of their own need. Hence, for commercial purposes, strain improvement is mandatory to obtain mutant bacteria with increased enzyme production.

Among all natural proteases obtained from *L. enzymogenes*, Lysyl endopeptidase which specifically cuts proteins from the Lys-I-Xaa bond attracts more attention in biotechnology [9,10]. In addition to the lysine specificity, the most important feature of the enzyme is maintaining activity in the presence of denaturing agents such as urea and sodium dodecyl sulfate (SDS). These characteristics made the enzyme very useful in proteomics techniques including sequencing, analyzing, and determining protein structures (peptide mapping) and also in-gel digestion protocols [11].

Mutagenesis is the most commonly used approach for strain improvement that can be induced randomly or site-specific [12,13]. Random mutagenesis is a process in which, by using chemical and physical mutagenic agents, changes are made in the characteristics of products produced by genes.

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Sometimes the product is not produced at all or abnormal products are produced. But sometimes this process leads to increased harvest production compared to the non-mutant or parent strain [14,15]. In this regard, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as a classical DNA alkylating agent, is one of the most effective chemical mutagens that converts GC to AT in DNA strands, so it can generate a large number of mutants under optimum conditions [16]. Therefore, in the present study, random mutagenesis using NTG as a chemical mutagen was employed to enhance protease production of the *L. enzymogenes* strain. The isolated mutants with hyper proteolytic activity will be further analyzed in order to obtain industrial strain for the production of Lysyl endopeptidase.

Material and method

Microorganism and growth media

The bacterial strain *Lysobacter enzymogenes* 29486™ was purchased from American Type Culture Collection (ATCC, USA). The bacterium was revived in brain heart infusion broth (Merck, Germany) and incubated at 33 ± 1 °C based on ATCC recommendation. The medium was enriched for growth rate and protease production by adding 1% glucose, 0.01% of each mono-and dibasic potassium phosphate, and 0.02% magnesium sulfate, based on a previous study performed by Kuhlman, et al. [17].

Chemical mutagenesis

Inoculums preparation: A single colony of *L. enzymogenes* bacterial cells was transferred to 5 ml of nutrient broth and maintained at 33 ± 1 ºC with shaking at 180 rpm to reach an appropriate optical density (OD_{600nm}), and then the inoculation was done by adding this sample to 50 ml of nutrient broth and incubated for an overnight.

NTG stock preparation

In the present study, NTG as a mutagenic substance was used to enhance the production of protease enzymes in *L.enzymogenes*. Initially, a stock solution of NTG was prepared by dissolving 0.01 g of NTG powder (TCI, Tokyo, Japan) in 1ml of dimethyl sulfoxide (DMSO, Merck, Germany). Various concentrations of NTG were made by diluting the primary stock in different amounts of 0.05M sodium phosphate buffer and then passing through a 0.22 µm syringe filter.

Mutagenesis procedure

Firstly, the OD_{600nm} of the inoculated culture was measured and adjusted to 0.1 by 0.05M phosphate buffer saline (PBS). Then, the bacterial pellet was separated by centrifuging at 10,000 rpm for 5 min, washing 3 times with PBS, and then re-suspending in PBS. The suspension was treated with various concentrations of NTG (100, 150, and 200 µg/ml) for 20 and 40 min at 33 ± 1 ºC under shaking at 130 pm. After incubation times, the treated cells were separated by centrifugation at 10,000 rpm for 10 min and washed 3 times with PBS to discard NTG. Finally, 1 ml of fresh nutrient broth was added to treated bacterial cells and incubated at 33 ± 1 ºC under shaking at 130 rpm overnight. The procedure has been used was the modified methods of Lynn, et al. [18] and Zambare [19].

Assessing bacterial viability after NTG treatment

The bacterial colony-forming units (CFU) counting was exerted to evaluate the effect of NTG on the survival rate of bacteria after treatments. For this purpose, the OD_{600nm} of wild-type (as control) and NTG-treated bacterial suspensions were adjusted to 1 and then serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were prepared in PBS. Then, 0.1 ml of diluted mutated bacteria were cultured on a nutrient agar medium and incubated at 33 ± 1 ºC for 48 h. For each sample, the dilutions which resulted in 30-300 numbers of colonies on the solid media were chosen for colony counting [20].

Screening methods

The initial screening stage is performed to select colonies with a clear hydrolysis zone of casein as substrate based on a previous study from Yokota, et al.[21], with some modifications. Single mutated clones were selected randomly from nutrient agar medium and cultured on skim milk agar plates (Merck, Germany). This medium was applied to isolate the mutants with proteolytic activity by exhibiting a zone of clearance around colonies. Then, in the secondary screening, the overnight cultures were prepared for each randomly selected NTG-treated bacterium and 20 µl of bacterial suspensions were transferred at the center of wells which were made in the 2.5% of skimmed milk agar. On each plate, one well devoted to wild-type bacterium and one for nutrient broth without bacteria, as a negative control. The plates were incubated at 33 ± 1 ºC for 48 h, and then the diameter of the clear zone around each mutated colony was measured and compared to the clear zone of the wild type. The procedure was done again in triplicates for those mutants that showed larger zones' diameter than the wild-type strain. Then, the bacterial supernatants were examined and compared to the wild-type protease activity. The statistical analysis of casein hydrolysis zone increment of mutant isolates in contrast to the wild-type bacteria was performed using a one-way ANOVA test using Graph Pad Prism software, version 9.0.1. A p-value of less than 0.05 was considered a significant difference between the groups.

Proteolytic enzyme activity

Enzyme activity was further assessed by a modified version of Anson's method [22] which measures the release of tyrosine from digested casein by proteases present in bacterial supernatant. For supernatant collection, an overnight culture of each selected mutant was added into a 250ml-Erlenmeyer flask containing 50ml of enzyme production medium and incubated under shaking for 2, 8, 10, 16, 24, and 48 h. After each incubation time, the cell-free supernatants were collected by centrifugation at 6000 rpm for 10 min. Then, 1 ml of supernatant was transferred into 2 ml casein solution (0.65% W/V in 0.05 M phosphate buffer, pH: 7) and the mixture was maintained for 30 min at 37 °C. The enzyme-substrate reaction was terminated by adding 2 ml of trichloroacetic acid (TCA, 110 mm, Merck, Germany) to the mixture and incubating for

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30 min at 37 °C. In the following, the mixture was centrifuged at 6000 rpm for 10 min, the pellet was discarded, and 1 ml of supernatant was mixed with 5 ml of $NaCO_3$ (0.5 M) and 1 ml of Folin–Ciocalteu reagent (0.5 M, Sigma, USA) and hold for 20 min at room temperature in a dark place. After the incubation period, OD was measured at 660 nm. The Enzyme unit was calculated by plotting a tyrosine standard curve (10–500 µg) and expressed as the amount of enzyme that released 1 $\mu g.ml^{-1}$ of tyrosine/min according to the test condition (Zambare 2010; Anson 1938) [19,21].

Results

Mutagenesis and determination of survival rate

Random mutagenesis by NTG was employed to obtain mutant isolates with increased proteolytic activity. For this, wild-type strain was exposed to different concentrations of NTG at various time intervals. The viability percentage of mutants was measured by CFU counting for two concentrations (The highest and the lowest) of NTG (100 and 200 µg/ml) in 20 and 40 minutes exposure times. As shown in Table 1, the survival rate significantly decreased with an increasing dose of NTG, representing a dose-response pattern (Table 1). After 20 min exposure to 100 µg/ml of NTG, the survival rate decreased to 60.8 %, whereas the lowest survival rate was 31.1%, which was obtained after 40 min treatment with 200 µg/ml of NTG.

Screening of mutants on a skimmed milk agar plate

From NTG-treated bacteria, a number of colonies were randomly selected and streaked on 2.5% skimmed milk agar for primary screening. Based on the casein hydrolysis clearance zone, 30 mutants were selected for further investigation. As shown in figure 1, the proteolytic activity was disrupted in some of the mutants after NTG treatment which was excluded from the rest of the study (Figure 1A). Further screening was performed with the culture of 30 isolated mutants using the well diffusion method and carefully measuring the casein hydrolysis clearance zone (Figure 1B).

The secondary screening was performed on the mutated isolates which showed a larger zone of casein hydrolysis in contrast to the wild type. For this, supernatants were harvested from the logarithmic phase of the mutant and wild-type bacteria, and the casein hydrolysis zone diameter was measured and compared with the wild-type supernatant (all experiments were done as triplicates).

Among 30 examined strains, 11 mutated strains showed higher protease activity. However, based on the results demonstrated in Table 2, the clear zones around M4 (100 µg/ml for 20 min) and M10 (200 µg/ml for 40 min) mutants showed a statistically significant increment of casein hydrolysis zone in contrast to the wild type isolate; 22.07 and 21.45 mm, respectively vs. 17.04 in wile type. (One-way ANOVA test, p-value < 0.01; Table 2).

Determination of protease activity of selected mutants

During the secondary screening, both isolated mutants were grown in nutrient broth and the supernatants were collected during the logarithmic phase (OD_{600} = 0.4, and OD_{600} = 0.6, based on the results of a preliminary study the highest amount of protease was obtained during the logarithmic phase of *L. enzymogenes* growth) and they were cultured into the wells on 2.5% skimmed milk agar for 48 h. The comparison of hydrolysis zone diameters showed that there was no significant difference at OD_{600} = 0.4 between M4 mutants and wild type, whereas it was 1.17 times larger in M4 than wild types at OD_{600} = 0.6 (Table 3). However, the clearance zone for the M10 mutant was higher than the wild type in both ODs (1.25 times higher in OD_{600} = 0.4 and 1.10 times in OD_{600} = 0.6; Table 3).

To calculate the activity of the enzyme in units per milliliter, the supernatant of isolated mutants was examined using the Anson method and the results were calculated based on the standard tyrosine curve. Accordingly, the highest yield of protease was obtained in M10 mutants during the logarithmic phase compared to M4 and wild-type strains (246.235 and 270.71 U/ml in OD_{600} = 0.4 and OD_{600} = 0.6, respectively; Table 3). However, the proteolytic activity of M4 mutant was also higher than wild types (197.34 and 154.6 U/ml in OD_{600} = 0.4, and OD_{600} = 0.6, respectively; Table 3).

Table 1: The survival rate of *L. enzymogenes* after NTG treatment for different time intervals.

Bacterial Suspension	CFU/ml	Death Rate (%)
Wild type bacteria	74×10^5	0
Mutant bacteria (100µg/ml of NTG for 20 min)	45×10^5	39.2
Mutant bacteria (100µg/ml of NTG for 40 min)	32×10^5	52.8
Mutant bacteria (200 µg/ml of NTG for 20 min)	28×10^5	62.2
Mutant bacteria (200 µg/ml of NTG for 40 min)	23×10^5	68.9

CFU: Colony-Forming Unit; min: Minute

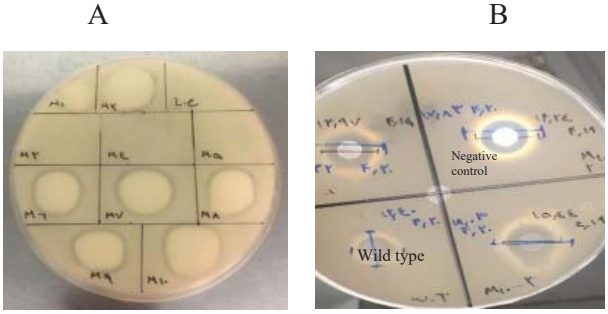

Figure 1: Initial screening step for selecting mutant L.enzymogenes strains. A) Primary selection of isolates by caseinolytic activity on skim milk agar. B) Well diffusion method in order to quantify protease activity. Culture media without bacteria was applied in the well located in the center of the plate as a negative control.

Discussion

Proteases are a large and extremely important group of enzymes in biology, medicine, and biotechnology. Microorganisms are a good source of enzymes with high diversity in biochemical properties as well as a better ability for genetic manipulation [1]. *Lysobacter enzymogenes* is a well-known gram-negative bacterium that produces different extracellular enzymes, especially proteases [23]. The most important of its

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Table 2: The isolated mutants from proteolytic screening on skim milk agar.

Treatment Time (min)	NTG concentration (µg/ml)	Mutants	Diameter of the clear zone (mm) ± SD	Increment relative to the wild type
0	0	Wild type	17.04 ± 0.3	--
		M1	17.64 ± 0.2	1.03
	100	**M4**	**22.07 ± 0.15**	**1.29**
		M5	18.13 ± 0.2	1.07
20	150	M3	19.01 ± 0.3	1.12
		M5	17.82 ± 0.15	1.04
	200	M3	17.95 ± 0.25	1.05
	100	M9	18.03 ± 0.21	1.05
	150	M2	17.92 ± 0.18	1.05
40		M4	18.08 ± 0.26	1.06
	200	M4	17.91 ±0.22	1.05
		M10	**21.45 ± 0.24**	**1.25**

Min: Minute; mm: Millimeter. The selected mutant isolates were indicated as bold.

Table 3: The comparison between extracellular protease activities in wild-type and isolated mutants.

Bacteria	OD_{600nm}	The diameter of the clearance zone of supernatant(mm)	Protease activity (IU/ml)	Increment relative to the wild type
	0.4	17.07 ± 0.18	99.6	-
Wild type				
	0.6	16.83 ± 0.24	105.71	-
	0.4	17.18 ± 0.27	154.6	**1.55**
M4	0.6	19.71 ± 0.32	197.34	**1.86**
	0.4	21.45 ± 0.31	246.235	**2.65**
M10	0.6	18.68 ± 0.25	270.71	**2.56**

min: Minute; mm: Millimeter; IU: International Unit.

enzymes is endopeptidase Lys-C, which is extensively used in biotechnological techniques such as primary-structure analysis, peptide mapping, in-gel digestion, and cleavage of fusion proteins in recent years [9]. However, the low yield of enzyme production from its source is the main concern for large-scale production. In this regard, modification of enzyme production pathway in such producer microorganisms is a crucial method to elevating production rate [24,25]. Random mutagenesis by chemical mutagens is one of the traditional approaches that is widely applied for strain improvement [26]. NTG is a very efficient mutagen from DNA alkylating agents which showed mutagenic effect by adding methyl to guanine and converting guanine to O6 methyl guanine [16].

Several previous studies exerted NTG mutagenesis to improve the production of various enzymes and metabolites in different microorganisms. Random mutagenesis by NTG on *B. subtilis* resulted in overproduction of Poly-γ-glutamic acid higher than the wild type in five selected isolates [27]. Based on such documentation, NTG mutagenesis was performed in the present study on *L. enzymogenes* to obtain mutants with increased proteolytic activity. To produce enough mutants with a high viability rate for screening, the fresh culture of *L. enzymogenes* was treated with different concentrations of NTG at various time intervals and the survival rate was evaluated by CFU counting. Based on the results, the survival percentage was decreased with elevating the NTG concentration and the exposure time. So that the lowest survival rate was 30.1% which obtain by treating bacterial suspension with 200 µg/ml of NTG after 40 min. The same trend of survivability reduction due to a higher concentration of the mutagenic agent has also been reported. In a study performed by Arshad, et al. [28], it was reported that treatment of *Escherichia coli* strains with NTG exhibited a dose and time-dependent pattern.

Another study conducted by Zambare in 2010 aimed to increase protease activity in *Trichoderma reesei* MTCC-3929 using a combination of UV and NTG mutagen. Mutant isolates were screened as protease producers based on the clearance zone diameter on the skimmed milk agar medium. Finally, a mutant called NTG-17 was found with a 2.6-fold increment of protease activity in contrast to the wild type. In the same manner, Zeng, et al. [29] reported that the isolate *NCU116 produces approximately 31.9% fold more proteinase over the parental strain* by combining ultraviolet irradiation and NTG treatment for mutagenesis.

In the present study, to isolate mutants with enhanced proteolytic activity, treated colonies were randomly streaked on 2.5% skim milk agar and all mutants with the ability to hydrolyze casein were selected. As a result of the screening procedure, 11 mutants that showed increased proteolytic activity in contrast to the wild type were selected. Among them, M4 (100 µg/ml for 20 min) and M10 (200 µg/ml for 40 min) mutants exhibited maximum proteolytic activity (Table 2). Since *L. enzy mogenes* normally released proteases during the logarithmic phase of the growth, supernatants of M4 and M10 were collected and analyzed during the logarithmic phase. Based on the casein hydrolysis zone, M10 mutant showed the highest protease activity which was 1.45- fold increment in OD_{600} = 0.4 in comparison with the wild type. Moreover, the unit of enzyme activity was qualified by a modified version of the Anson method which in the highest extracellular protease activity was obtained in M10 mutants during logarithmic phase compared to M4 and wild type strains which were 2.65 and 2.56 fold of wild type in OD_{600} = 0.4 and 0.6, respectively.

The earlier studies showed that the use of combinational mutagenesis by NTG and a physical mutagen made mutation more efficient. Accordingly, the combinational mutagenesis on the *Bacillus pumilus* strain created a mutant strain with increased protease activity (6000 U/ml in contrast to 1200 U/ml of wild type) [30]. Similarly, another study was conducted by Muhammad Nadeem, et al. [31]. They found that applying physical and chemical mutagenesis showed 1.4 fold higher protease activity in the pre-optimized growth medium than the parent strain. In our previous studies, physical mutagenesis using UV radiation and cold atmospheric plasma on the *L. enzymogenes* strain leads to isolating mutant strains with enhanced proteolytic activity with the highest ratio of 1.91 [32,33]. Therefore, it is predictable that the combination of UV and NTG could be also efficient for enhancing the proteolytic activity of *L. enzymogenes* which should be assessed in future studies.

Conclusion

Taken together, the results of the present study suggested that using NTG as a chemical mutagen can be a suitable method for generating mutants with higher proteolytic activity.

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However, the number of improved mutants and the amount of proteolytic activity increment in this method were relatively low; hence it seems that the use of NTG in combination with other mutagens would be a more potent way for the strain improvement.

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